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## Exhibit L

# Recombinant Adeno-Associated Virus-Mediated Gene Transfer Into Hematopoietic Progenitor Cells

By Stacey Goodman, Xiao Xiao, Robert E. Donahue, Austine Moulton, Jeffrey Miller, Christopher Walsh, Neal S. Young, Richard J. Samulski, and Arthur W. Nienhuis

Recombinant adeno-associated viruses (rAAV) containing only the inverted terminal repeats (ITR) from the wild-type virus are capable of stable integration into the host cell genome, and expression of inserted genes in cultured cells. We have now defined the ability of rAAV to introduce genes into primary hematopoietic progenitors. A vector was constructed containing the coding sequences for  $\beta$ -galactosidase ( $\beta$ -gal), including a nuclear localization signal, under the control of a strong viral promoter. Infectious vector particles were prepared by cotransfection of the vector plasmid with a second plasmid that contained the coding sequences for AAV proteins into adenovirus-infected human embryonic kidney cells. These vector preparations transferred and expressed the  $\beta$ -gal gene in human K562 erythroleukemia and Detroit 6 cells. Positive immunoselection yielded a popula-

tion of enriched CD34<sup>+</sup> cells that were transduced with the rAAV  $\beta$ -gal vector. Nuclear localized enzyme expression was documented in 80% to 70% of infected cells. Progenitor-derived colonies that developed after 2 weeks in clonogenic cultures were shown to have viral-associated DNA at an estimated copy number of 1 to 2 per cell using a semiquantitative polymerase chain reaction (PCR) method. Integration of AAV into hematopoietic progenitors was documented using wild-type virus, as its genome may integrate at a preferred site on chromosome 19. Our data suggest that rAAV will transfer and express genes in primitive hematopoietic progenitors with high frequency, and support the development of this vector system for therapeutic gene transfer.

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**T**HE ABILITY TO transfer genes into repopulating hematopoietic stem cells and to achieve regulated gene expression in lymphoid or myeloid lineages would create many therapeutic opportunities.<sup>1,2</sup> Several genetic diseases affect one or more derivatives of the hematopoietic stem cell, including the red blood cell disorders—sickle cell anemia and thalassemia, chronic granulomatous disease affecting phagocytic cells, various platelet disorders, and immunodeficiency diseases affecting lymphoid cells. One proposed approach to the treatment of acquired immunodeficiency syndrome (AIDS) is to introduce a genetic element into hematopoietic stem cells that renders lymphoid cells in which this element is expressed resistant to human immunodeficiency virus (HIV) infection.<sup>3</sup> Targeting gene expression to individual hematopoietic lineages will undoubtedly depend on the use of tissue-specific, *cis*-active DNA elements, about which much is currently being learned.<sup>4,5</sup> A major barrier to stem-cell-targeted gene therapy in humans has been the difficulty of introducing new genetic information into the quiescent stem-cell population.<sup>1,2,6</sup>

During the past decade, murine models have been developed for stem-cell-targeted gene transfer using retroviral vectors.<sup>1,6,7-11</sup> Vector preparations devoid of replication competent virus are derived from engineered producer clones, in which the proteins required for retroviral particle formation result from expression of viral structural genes that are part

of one or more transcriptional units.<sup>2,12</sup> A vector genome is introduced separately, and its expression generates the RNA genome needed for assembly of vector particles. Preparations containing 10<sup>6</sup> infectious particles/mL infect murine repopulating stem cells from animals pretreated with 5-fluorouracil given to activate the stem-cell population.<sup>8,11</sup> Stimulation of bone marrow cells *in vitro* with various cytokine combinations for 48 hours assures that some stem cells are in cell cycle when subsequently exposed to vector particles,<sup>10,11</sup> a condition necessary for integration of the retroviral vector genome.<sup>13,14</sup> Long-term reconstitution of recipient mice with genetically modified cells is regularly achieved, and gene expression has been documented for more than a year.<sup>11,15-18</sup> Unfortunately, the majority (60% to 70%) of murine bone marrow stem cells remain refractory to retroviral-mediated gene transfer with currently used protocols. Attempts have been made to apply lessons learned in the murine models in canine<sup>19,20</sup> or nonhuman primate models.<sup>21,22</sup> Persistent long-term gene expression has been documented in such large animal models, establishing the feasibility of this general approach, although the frequency of gene insertion into stem cells remains disappointingly low. Only 1% to 3% of primate stem cells are susceptible to retroviral-mediated gene transfer.<sup>1,2</sup>

Among the various approaches that are being explored to improve the efficiency of gene transfer into stem cells is the use of adeno-associated viral (AAV) vectors,<sup>2,23</sup> in hopes of developing a safe and efficient viral vector for human gene therapy. AAV is a dependent parvovirus; it requires coinfection with another virus (either adenovirus or certain members of the herpes virus group) for productive infection of cultured cells. In lytic infection, AAV DNA replicates as a 4.7-kilobase double-stranded molecule, and is packaged into virions as linear single strands of both polarities.<sup>23</sup> In the absence of coinfection with helper virus, the AAV genome integrates via its termini into the host genome in a site-specific manner, and remains latent until the cell is infected with helper virus<sup>24-26</sup> when the AAV DNA is rescued, replicates, and establishes a productive infection.<sup>23</sup> Several features of AAV make it an attractive vector: (1) the virus is ubiquitous in

From the Clinical Hematology Branch, National Heart, Lung and Blood Institute, Bethesda, MD; and the Department of Biology, University of Pittsburgh, Pittsburgh, PA.

Submitted December 8, 1993; accepted May 4, 1994.

Previously published in abstract form, *Blood* 80:167a, 1992.

Address reprint requests to Arthur W. Nienhuis, MD, St Jude Children's Research Hospital, 332 N Lauderdale, Memphis, TN 38101.

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0006-4971/94/8405-0030\$3.00/0

humans, (2) AAV particles can be concentrated to titers exceeding  $10^9$  infectious units per milliliter, (3) AAV has a broad host range for infectivity (human, monkey, mouse, etc) when coinfecting with the appropriate helper virus, and (4) it is a completely nonpathogenic integrating virus.

Current AAV vectors use an infectious recombinant clone in which 96% of the viral genome is replaced with the gene of choice, retaining only the viral terminal repeat sequences of 145 nucleotides<sup>2,23,29,30</sup> (X. Xiao and R.J. Samulski, unpublished observations). Packaging systems have been developed in which recombinant vector genomes generate vector preparations devoid of wild-type AAV.<sup>29</sup> These preparations, which require removal of the adenovirus helper, are thought to be capable of infecting quiescent cells.<sup>23</sup> Gene transfer and regulated expression has been achieved using a recombinant AAV (rAAV) vector to insert the human gamma-globin gene into human erythroleukemia cells.<sup>31,32</sup>

In working toward the ultimate goal of stem-cell-targeted gene transfer, we have now explored the ability of rAAV to achieve gene transfer into primate clonogenic hematopoietic progenitors. A rAAV containing the  $\beta$ -galactosidase ( $\beta$ -gal) gene was used, providing a convenient means to assay for transduction and gene expression in cell monolayers or in single-cell suspensions. Semiquantitative polymerase chain reaction (PCR) analysis was used to evaluate DNA from progenitor-derived colonies for evidence of gene transfer. Site-preferred integration into chromosome 19, a feature of latent infection with wild-type AAV, is not generally observed with rAAV. Therefore, in our experiments, we used wild-type AAV to evaluate integration in primary hematopoietic cells.

## MATERIALS AND METHODS

**Viruses and cells.** Wild-type human adenovirus 5 (Ad5) was obtained from the American Type Culture Collection (ATCC), Rockville, MD. The human bladder carcinoma cell line, 5637, was also obtained from the ATCC. Detroit 6,<sup>29</sup> 293,<sup>33</sup> and 562 cells<sup>34</sup> were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS), 2 mmol/L glutamine, 100 U/mL penicillin, and 0.1 mg/mL streptomycin. A cell line containing a single integrated copy of the  $\beta$ -gal gene as part of a proviral genome (G1BgSVNa-clone 29), obtained from Genetic Therapy (Gaithersburg, MD), was maintained in the same medium. 5637 cells were maintained in RPMI 1640 containing 10% FCS, 2 mmol/L glutamine, 100 U/mL penicillin, and 0.1 mg/mL streptomycin. The cells were grown to confluence, transferred into DMEM containing 10% FCS, 2 mmol/L glutamine, 100 U/mL penicillin, and 0.1 mg/mL streptomycin, and the 5637 cell-conditioned media was harvested after 7 days.

**Construction of the rAAV- $\beta$ -Gal vector.** The plasmid pNLacF containing the coding sequences for LacZ ( $\beta$ -gal), modified to incorporate a eukaryotic translation initiation codon, an N-terminal nuclear localization signal,<sup>35</sup> and the RNA-processing signals from the mouse protamine-1 (mP-1) gene,<sup>36</sup> was obtained from Jacques Pershon (Immunex Co, Seattle, WA). The CMV E1-A promoter within an *XhoI*-*PstI* fragment was inserted as a blunted fragment into a blunted *XbaI* site 5' to the LacZ coding sequences. The entire cassette containing the CMV promoter,  $\beta$ -gal coding sequences, and mP-1 RNA-processing signals was inserted between the *NsiI*-*SnaBI* sites of pDX11<sup>37</sup> to create the plasmid pAB-11. pDX11 had been

derived by subcloning the rAAV genome from pd113-94<sup>37</sup> into the *PstI* site of pGEM3A (Promega, Madison, WI).

**Generation of AAV and rAAV vectors.** AAV type 2 was obtained from ATCC, and maintained by infecting either 293 or HeLa cells; helper function was provided by Ad5 strain dl309 (a gift from T. Shenk, Princeton University), used at a multiplicity of infection (MOI) of 10. At 48 hours postinfection, the cells were collected with the medium, frozen, and thawed three times. After low-speed centrifugation, the cell lysate containing wild-type AAV was heated to 55°C for 1 hour to inactivate the adenovirus, aliquoted, and stored at -20°C for use. The titer of the AAV virus stock was determined indirectly either by immunostaining of infected cells with antipapillid antibodies or by quantitative Southern blot analysis. For preparation of rAAV, 60% to 80% confluent 293 cells were infected with adenovirus type 5 at an MOI of 5 to 10 as previously described.<sup>29</sup> rAAV viral stocks were generated by subsequent calcium phosphate cotransfection of 10  $\mu$ g of plasmid pAB11 and 10  $\mu$ g helper plasmid (pAAV/Ad), 2 to 4 hours after adenoviral infection. Cells were harvested 48 to 72 hours posttransfection, frozen and thawed four times, and centrifuged to remove debris. Inactivation of adenovirus was achieved by heating to 55°C for 30 minutes. Heating of the viral preparations eliminated infectious adenovirus, as no cytopathic effect was observed over 7 to 10 days after exposure of 293 or Detroit 6 cells to lysate diluted 1:1. For some preparations, in an effort to achieve a higher viral titer, the plasmid DNA was introduced by lipofection using liposomes prepared according to protocols provided by the vendor, GIBCO (Grand Island, NY). For these preparations, the cells were lysed by sonication in DMEM-10% FCS or phosphate-buffered saline (PBS), clarified by centrifugation, and stored at 4°C.

**Histochemical staining for  $\beta$ -Gal activity.** Aliquots of lysates (0.01 mL to 0.1 mL) were added to Detroit 6 cells at 60% to 80% confluency and allowed to incubate for 18 hours. Plates were washed, fixed with 2% formaldehyde and 0.2% glutaraldehyde for 5 minutes at 4°C, and stained for X-gal as previously described<sup>38</sup> for 36 hours. The cells were washed three times in PBS and the enzyme reaction developed in PBS containing 1 mg/mL of X-gal, 2 mmol/L MgCl<sub>2</sub>, 5 mmol/L potassium ferrocyanide, and 5 mmol/L potassium ferricyanide at 37°C for 24 to 36 hours. The cells were then washed in PBS. Cytospin preparations of suspension cells were processed identically after fixation for some experiments. Individual blue nuclei were counted to estimate the viral titer. For processing of fresh cells, 150  $\mu$ g of X-gal/mL was added to the culture medium and the incubation continued at 37°C in 5% CO<sub>2</sub> for 16 hours. Cytospin preparations were prepared using standard techniques.

**Purification of human and rhesus bone marrow progenitors.** Human bone marrow cells were obtained from normal volunteers after informed consent using a study protocol approved by the National Heart, Lung and Blood Institute Review Board. Bone marrow aspirates were obtained by standard clinical techniques. Low-density mononuclear cells were isolated by buoyant-density centrifugation (Ficoll-Hypaque), and CD34<sup>+</sup> cells were recovered by positive immunoselection using an avidin-biotin-conjugated column system (Ceprate LC) according to the manufacturer's instructions (Cell-Pro, Bothell, WA). Purity was estimated by flow cytometric analysis of the immunoselected CD34<sup>+</sup> cell population after restaining with an anti-CD34 antibody or mouse antihuman IgG2a (isotypic control) conjugated to phalloerythrin. Sixty percent to 90% of the recovered cells were CD34<sup>+</sup>. In addition, the starting mononuclear cell population and immunoselected CD34<sup>+</sup> cells were plated in clonogenic cultures in methylcellulose using standard conditions (see below). A 57- to 90-fold enrichment in colony-forming progenitors was achieved.

Rhesus bone marrow cells were obtained by aspiration and a mononuclear cell preparation prepared by buoyant-density centrifuga-

gation; CD34 selection was performed using an antibody (K6.1) that reacts with rhesus CD34 antigen by techniques previously described.<sup>39</sup> A 12- to 94-fold enrichment was achieved, as reflected by the concentration of clonogenic progenitors in the starting mononuclear versus the immunoselected cell population.

**Infection and culture of enriched progenitors.** CD34<sup>+</sup> immunoselected cells were incubated in suspension culture containing DMEM with 10% to 15% FCS, 2 mmol/L glutamine, 100 U/mL penicillin, 0.1 mg/mL streptomycin, 10 ng/mL interleukin (IL)-3, 50 ng/mL IL-6, and 100 ng/mL stem-cell factor (SCF). These cultures were exposed to no virus, wild-type AAV, or rAAV for 1 to 96 hours, at which time cells were harvested, washed, and aliquoted for morphologic analysis (cytoprep), methylcellulose assay, and, in some instances,  $\beta$ -gal activity analysis.

Human bone marrow mononuclear cells (10<sup>5</sup>/mL) and CD34<sup>+</sup> immunoselected cells (10<sup>5</sup>/mL) were plated in Iscove's methylcellulose medium (Terry Fox Laboratories, Vancouver, Canada) consisting of Iscove's media, 0.8% methyl cellulose, 30% FCS, 1% bovine serum albumin, and 10<sup>-4</sup> 2-mercaptoethanol, to which was added 10% 5637 cell-conditioned medium and 2 U/mL of erythropoietin. Cultures were maintained at 37°C in 5% CO<sub>2</sub> for 12 to 14 days. After scoring, colonies were processed individually or in pools for extraction of DNA. For certain experiments, IL-3 10 ng/mL, IL-6 50 ng/mL, and stem-cell factor (SCF) 100 ng/mL (provided by AMGEN, Thousand Oaks, California) were used in place of 5637 cell-conditioned medium. Culture of rhesus bone marrow cells before and after CD34 immunoselection in methylcellulose was as previously described.<sup>39</sup>

**DNA isolation and analysis by the PCR methodology.** For preparation of DNA from hematopoietic colonies, single colonies were plucked and placed into 50  $\mu$ L of diethylpyrocarbonate-treated water. Mineral oil was layered over the aqueous phase, and the samples were heated at 100°C for 10 minutes and cooled to 4°C. Proteinase K (Pro-K) was added to a final concentration of 400  $\mu$ g/mL, and the samples were incubated at 55°C for 90 minutes and then at 100°C for 5 minutes (to inactivate Pro-K) before cooling to 4°C. DNA, for use as controls, was isolated by standard techniques<sup>40</sup> from cultured cells or bone marrow mononuclear cells.

Standard PCR methodology for DNA analysis was performed using a kit provided by Perkin-Elmer/CETUS (Norwalk, CT) under the conditions specified by the manufacturer. Each 100- $\mu$ L reaction contained 10  $\mu$ L of the DNA preparation from individual or pooled colonies. [<sup>32</sup>P]dCTP (800 Ci/mmol) (Amersham-Searle, Arlington Heights, IL) was added in the amount of 0.2 to 0.5  $\mu$ L per reaction. Four hundred nanograms of each primer and 2.5 U of Taq DNA polymerase were also added to each 100- $\mu$ L reaction. The PCR cycles for DNA analysis were preceded by incubation at 95°C for 2 minutes, and the final cycle was followed by elongation at 72°C for 7 minutes. Twenty-four percent of each reaction mixture was analyzed on an 8% polyacrylamide gel that was processed for autoradiography as previously described.<sup>39</sup>

A set of primers based on the sequence of the mouse  $\beta$ -actin coding region were used to amplify the human  $\beta$ -actin sequences to yield a 232 bp fragment. The sequences were as follows: 5' primer, 5'-CATTTGATGGACTCCGGAGACGG-3'; and 3' primer, 5'-CATCTCCTGCTCGAAGTCTAGAGC-3'. The PCR was conducted for 25 cycles under the following conditions: 95°C for 1 minute, 58°C for 1 minute, and 72°C for 1.5 minutes. The sequences of the primer pair used to amplify a 314 bp segment of the rhesus  $\gamma$ -globin gene are as follows: 5' primer, 5'-GTTGGG-AGTGAAGAACTGC-3'; and 3' primer, 5'-TAGCCTCAGACTCTGTTTGG-3'. The PCR was conducted for 30 cycles under the following conditions: 94°C for 1 minute, 55°C for 1 minute, and 72°C for 1 minute. Another primer pair was used to amplify a 247-bp segment of the  $\beta$ -Gal gene. The sequences are as follows: 5'

primer, 5'-CTACACCAACGTAACCTATCCC-3'; and 3' primer, 5'-TTCTCGGCGCGTAAAAATGCG-3'. The PCR was conducted for 30 cycles under the following conditions: 94°C for 1 minute, 55°C for 1 minute, and 72°C for 1 minute.

**Integration site analysis.** The wild-type AAV genome was detected using the primer pair, 5'-GAACGCGCAGCCGCC and 5'-GCGCATCAGAATTGGGATTC, that give a 635-bp amplification product derived from the 5' end of the AAV genome. PCR amplification was performed for 25 to 30 cycles under the conditions described below. AAV integration into chromosome 19 was detected by PCR using nested primer pairs that flank an AAV-chromosome 19 junction<sup>24,25</sup>; Jus2 (AAV) -5' AGTAGCATGGCGGGT and Jus3 (chromosome 19) -5' CGCGCATAAGCCAGTAGAGCC. PCR was for 25 cycles using previously described reaction conditions with the following parameters: 1 minute at 94°C, 1 minute at 55°C, and 2 minutes at 73°C. Two percent of the amplification product was diluted into a new reaction mixture containing a set of nested primers with the following sequences: TR-1 (AAV), 5'-GGAATTC-AGGAACCCCTAGTGATGG; and CR-2 (chromosome 19), -5'-ACAATGGCCAGGGCCAGGCAG. The PCR parameters were the same as for the first amplification, and 25 cycles were completed. The products were resolved on a 1% agarose gel, transferred to Hybond N+ paper (Amersham), and probed with a previously cloned junction fragment<sup>25</sup> labeled by Amersham Megaprime DNA labeling system. For molecular cloning of amplified junction fragments, the two nested primers from the second amplification, TR-1 and CR-2, were modified by incorporation of an *Eco*RI or *Bam*HI restriction site, respectively. After amplification, the products were restricted with these enzymes, purified on a 1% agarose gel, and subcloned into plasmids, pUC18 and pUC19, by standard methods. Sequencing was performed by the chain-termination method using a kit obtained from Promega.

## RESULTS

**rAAV-mediated gene transfer and expression of the  $\beta$ -gal gene.** An AAV vector carrying the  $\beta$ -gal reporter gene was constructed to assay rAAV's transduction efficiency in various cell types. Addition of vector preparations at an MOI of 1 or greater to subconfluent Detroit 6 cells resulted in expression of the  $\beta$ -gal gene in most cells within 18 hours (Fig 1A). The viral titers were estimated by adding serial dilutions to a fixed number of cells in each plate; individual preparations ranged from 10<sup>4</sup> to 10<sup>6</sup> infectious particles/mL. Similar results were obtained with vector lysates derived by CaPO<sub>4</sub>-mediated gene transfer or by lipofection of the vector and helper (pAAV/Ad) plasmids; the titers of the two types of preparations did not vary in a consistent way. When the Detroit 6 cells were split twice and allowed to become confluent again, a much smaller proportion of cells expressed the  $\beta$ -gal gene. Expressing cells were clustered (Fig 1B), suggesting viral integration shortly after infection in a single cell that gave rise to a clone of expressing progeny. The disparity between the proportion of cells expressing the  $\beta$ -gal gene shortly after viral exposure, and the number exhibiting stable expression, suggest that viral uptake and initial gene expression may occur more efficiently than the integration step that leads to persistence of the rAAV genome. Retention of rAAV as an episome in the cluster of cells seems less likely, as most initially expressing cells had lost signal. Because the cells had been split twice before analysis, infection by an undispersed aggregate of virus seems unlikely, but cannot be completely ruled out. The frequency of positive

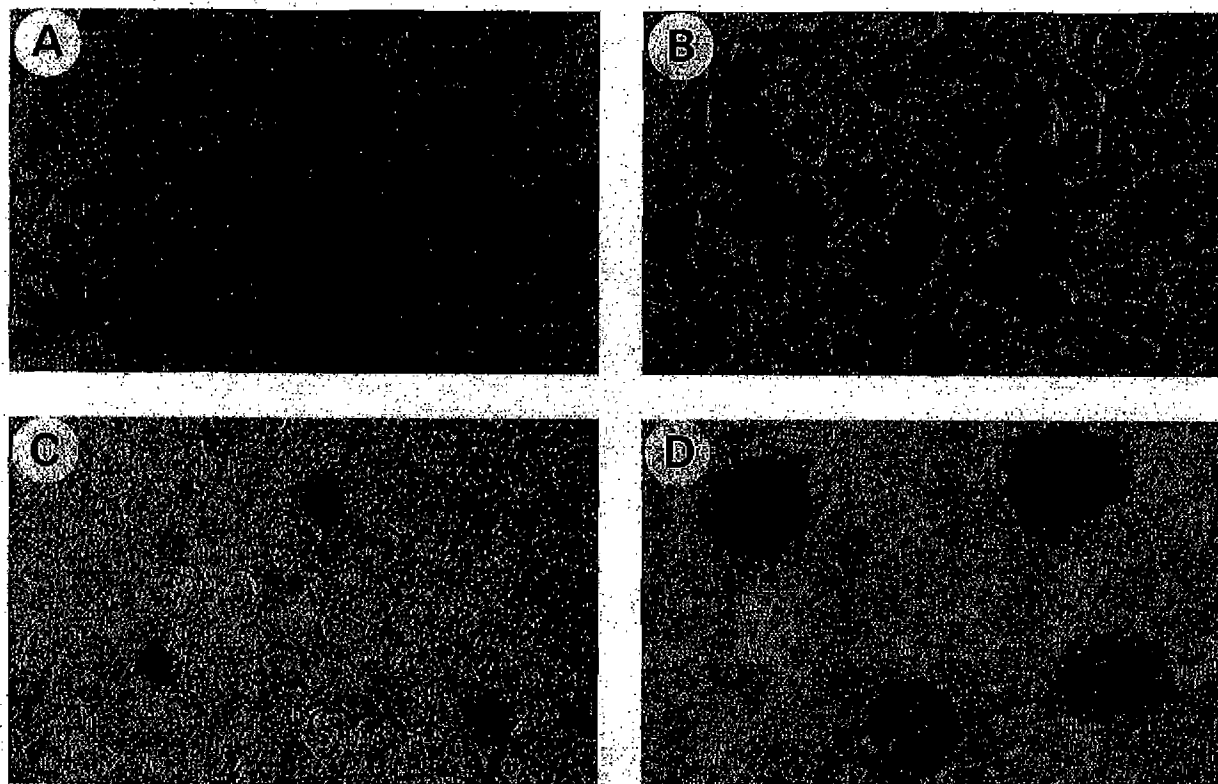


Fig 1. Expression of the  $\beta$ -gal gene after rAAV-mediated gene transfer. (A) Titration of viral preparations on Detroit 6 (D6) cells. D6 cells at 60% to 80% confluency in a 3.5-cm plate were infected by adding 100  $\mu$ L of virus preparation to 1 mL of medium. After 18 hours, the cells were fixed, washed, and stained for 36 hours as described in Materials and Methods. (B) Persistent expression of the  $\beta$ -gal gene in D6 cells after rAAV-mediated gene transfer. D6 cells were infected by exposure to virus for 12 hours and passaged twice over a period of 7 days. Occasional foci of cells staining positively for  $\beta$ -gal were identified after in situ fixation and staining of the cells. (C) rAAV-mediated transfer of the  $\beta$ -gal gene into human erythroleukemia cells. K562 cells at a concentration of  $2 \times 10^5$  cells/mL were incubated at a multiplicity of infection of approximately one with rAAV- $\beta$ -Gal for 48 hours at 37°. The cells were washed and cultured in vitro for an additional 5 days. A cytospin preparation was made, fixed, and stained as described in Materials and Methods. (D) Transfer and expression of the  $\beta$ -gal gene in human CD34<sup>+</sup> progenitors. Progenitors were enriched 81-fold by CD34 immunoselection, as determined by clonogenic assay.  $5 \times 10^5$  cells were incubated in 1 mL of bone marrow cell culture medium and 1 mL of virus preparation medium for 48 hours. Six hundred microliters of cell suspension was mixed with 200  $\mu$ L of the X-gal stain and the culture continued for 16 hours. A cytospin preparation was then prepared.

clusters approximated the frequency with which we previously recovered G418 resistance colonies with vectors containing the neomycin phosphotransferase (*neo<sup>R</sup>*) gene<sup>2,23</sup> supporting our interpretation that the rAAV  $\beta$ -gal genome has integrated in a small proportion of initially infected Detroit 6 cells (Fig 1B).

Transduction of human erythroleukemia cells was also achieved with the rAAV- $\beta$ -gal vector. Approximately 2% to 3% of cells exposed briefly to the virus at an MOI of approximately 1 expressed the  $\beta$ -gal gene 5 days later (Fig 1C). Similarly, exposure of CD34<sup>+</sup> immunoselected human progenitor cells to the virus for 3 days at an MOI of 1 to 10 resulted in expression of the  $\beta$ -gal gene in 60% to 70% of the cells. Control K562 or CD34<sup>+</sup> selected cells showed no nuclear staining; low-level, nonspecific cytoplasmic staining was avoided by maintaining the pH of the washing buffer at greater than 7.5. These data established that viral uptake and rAAV-mediated gene expression could be achieved at high efficiency in hematopoietic cells.

*rAAV- $\beta$ -Gal-mediated gene transfer into primate clono-*

*genic hematopoietic progenitors.* Attempts to detect  $\beta$ -gal activity in intact mature colonies were confounded by the fact that nuclear and cytoplasmic activities could not be distinguished in intact colonies and background staining was variably high. Hence, we developed a quantitative PCR assay to detect the rAAV genome in individual colonies. A cell line having one integrated copy of the  $\beta$ -gal gene per cell was used as a control. Conditions were found for which the signal intensity generated with the  $\beta$ -gal primers was proportional to the concentration of the rAAV genome over a broad range of total DNA input (Fig 2). The  $\beta$ -actin amplification could be used to verify that DNA from individual colonies gave a readily detectable PCR product and, by comparing its intensity to that of the  $\beta$ -gal PCR product, we could estimate the copy number of the rAAV genome.

Shown in Fig 3 is the application of this assay to the analysis of colonies derived from human progenitors exposed to an rAAV virus preparation, after heat inactivation of adenovirus, at an MOI of approximately 1 for 24 hours. After the infection interval was completed, the cells were

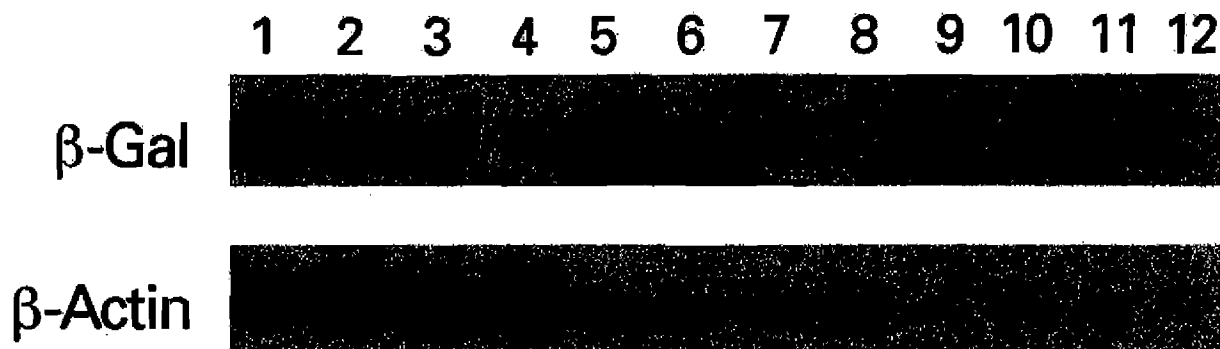


Fig 2. Calibration and sensitivity of the PCR reactions for detection of  $\beta$ -gal and  $\beta$ -actin coding sequences. Lanes 1 through 4 contain 1,000 ng of total DNA, lanes 5 through 8 contain 100 ng, and lanes 9 through 12 contain 10 ng. Each lane contains human DNA or mouse DNA from a cell line containing one integrated copy of the  $\beta$ -gal gene or mixtures thereof as follows: lane 1, 1,000 ng  $\beta$ -gal DNA; lane 2, 900 human DNA and 100 ng  $\beta$ -gal DNA; lane 3, 990 ng human DNA and 10 ng  $\beta$ -gal DNA; lane 4, 1,000 ng human DNA; lane 5, 100 ng  $\beta$ -gal DNA; lane 6, 90 ng human DNA and 10 ng  $\beta$ -gal DNA; lane 7, 99 ng human DNA and 1 ng  $\beta$ -gal DNA; lane 8, 100 ng human DNA; lane 9, 10 ng mouse  $\beta$ -gal DNA; lane 10, 9 ng human DNA and 1 ng  $\beta$ -gal DNA; lane 11, 9.9 ng human DNA and 0.1 ng  $\beta$ -gal DNA; lane 12, 10 ng human DNA. The  $\beta$ -actin coding sequences in mouse and human DNA amplified equivalently. The actin PCR product was generated with 25 cycles and visualized with a 20-minute exposure, whereas the  $\beta$ -gal-amplified PCR products were generated with 30 cycles and visualized with a 30-minute exposure.

plated in methylcellulose under standard conditions. Individual colonies were plucked when mature at 12 to 14 days. DNA from seven colonies gave an amplified  $\beta$ -gal signal of variable intensity, but in the range comparable to that of the  $\beta$ -actin control, suggesting that the viral genome was present at approximately single-copy equivalence in all cells of the colony. Four colonies were negative and one indeterminate (a  $\beta$ -actin signal was not generated from its DNA).

Shown in Fig 4 is an analogous experiment with CD34<sup>+</sup> immunoselected rhesus hematopoietic progenitors infected with a vector preparation that had been heated for 1 hour at 55°C to inactivate the adenovirus. At a low MOI, heat inactivation of the adenovirus appeared to enhance the frequency of transfer of the rAAV genome (data not shown). Results are shown for 15 colonies. Five were negative, five

gave a signal intensity approximately 50% of that of  $\gamma$ -globin, and the remaining five gave equivalent signal intensities with the two primer pairs. These data suggested that the proviral copy number may vary among colonies; and raised the possibility that integration occurs in only a proportion of the initial cells derived from a single progenitor.

*Integration of wild-type AAV into chromosome 19 in hematopoietic progenitors.* Wild-type<sup>24,28</sup> but not recombinant AAV<sup>31</sup> integrates into a preferred region on chromosome 19. To assess site-specific integration in primary hematopoietic progenitors, we exposed human CD34<sup>+</sup> immunoselected cells to wild-type virus preparations at high MOI (100 to 1,000). After culture for 36 hours in the presence of virus, progenitors were plated in methylcellulose and the colonies allowed to mature over 12 to 14 days. DNA

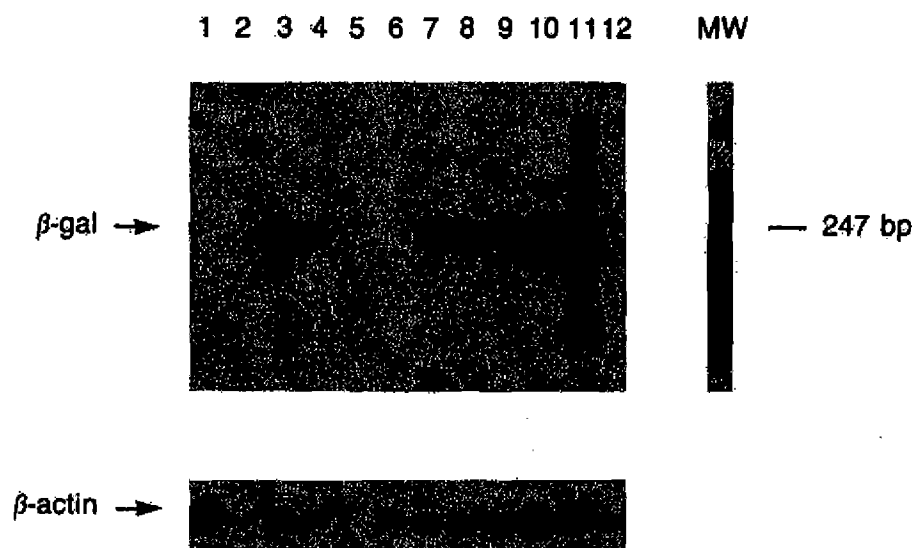
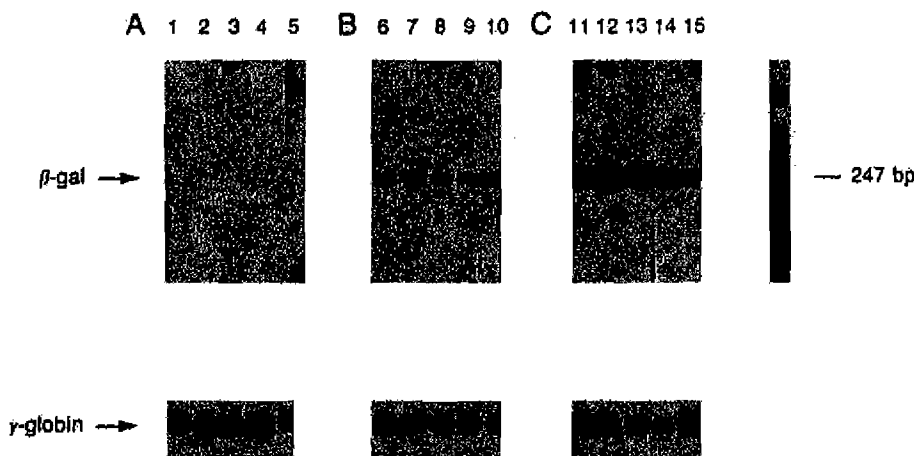


Fig 3. Detection of the rAAV- $\beta$ -Gal genome in individual human hematopoietic progenitor-derived colonies. DNA from 12 separate colonies were analyzed by PCR with primers specific for the  $\beta$ -gal or the  $\beta$ -actin coding sequences. One colony (5) was indeterminate, as the  $\beta$ -actin signal was not detectable. Seven of the remaining 11 colonies contained  $\beta$ -gal coding sequences and four were scored as negative.

**Fig 4.** Detection of the rAAV- $\beta$ -gal genome in hematopoietic colonies derived from rhesus progenitors. Shown are the results from 15 colonies in which the control primer pair for the rhesus  $\gamma$ -globin gene gave equivalent signals. (A) Five colonies lacking the  $\beta$ -gal coding sequences. (B) DNA from five colonies that gave a  $\beta$ -gal signal of intermediate intensity. (C) Five colonies in which the  $\beta$ -gal signal was equivalent or greater than that derived from the  $\gamma$ -globin gene.



from the majority of colonies (70% to 80%) generated an amplification product with a primer pair specific for the AAV genome of intensity comparable to, or greater than, that achieved with the  $\beta$ -actin primers (Fig 5). Because of the high estimated MOI, the potential that residual virus contributed to the signal for some colonies cannot be excluded. All colonies were derived from a single donor; the negative colonies establish that this individual was not harboring a latent AAV infection in bone marrow.

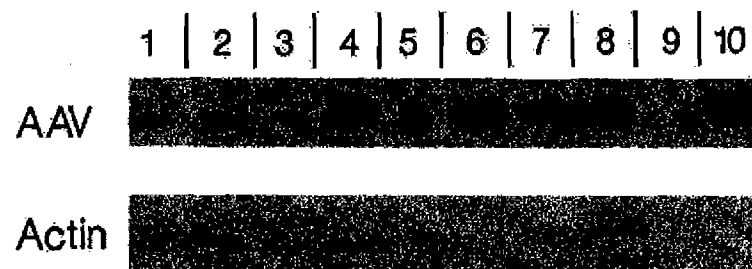
DNA from pools of 40 to 80 colonies or DNA from individual colonies were analyzed using two sets of nested primers that span the potential junction between the AAV genome and chromosome 19 DNA sequences. The pools and all of the colonies gave positive signals when analyzed for wild-type AAV DNA sequences by PCR. Duplicate samples from one pool gave a positive signal on the integration site analyses (Fig 6). These PCR-amplified products were subcloned into a bacterial plasmid and the sequence determined. A novel junction was defined between the truncated inverted terminal repeat of AAV and chromosome 19 (Fig 7). DNA from four of 27 individual colonies, all of which were positive for wild-type AAV sequence, gave a junction amplification product that annealed to chromosome 19 and AAV genome probes. Three of these were also cloned and sequenced and shown to contain a novel junction between chromosome 19 and the AAV inverted terminal repeat (data not shown). These data suggested that association of wild-type viral DNA with progenitor-derived colonies occurs with high frequency,

but that site-specific integration of the AAV genome into chromosome 19 is relatively rare.

#### DISCUSSION

These experiments were designed to evaluate the potential of rAAV to transcribe and express genes in primary hematopoietic progenitor cells. A high frequency of gene transfer and expression of the  $\beta$ -gal reporter gene was observed in progenitor cells, highly purified by positive immunoselection. A large proportion of colonies derived from these progenitors contained rAAV DNA at a concentration equivalent to one to two copies of the viral genome per cell. These data establish the potential of rAAV to infect human hematopoietic progenitors, but the efficiency of stable integration of the vector genome was not determined. Previous work has established that wild-type AAV will integrate into a preferred site on chromosome 19 in tissue culture cells.<sup>25-28</sup> To address the issue of AAV integration in hematopoietic progenitor cells, we tested wild-type AAV for targeted integration into chromosome 19 using a PCR-based assay. While we documented integration of the AAV genome into the preferred site on chromosome 19, this occurrence seemed to be a relatively rare event.

Because the AAV vector genome is packaged as a single-stranded DNA molecule, expression of the  $\beta$ -gal gene from the vector, as demonstrated in these experiments, argues for replication of the complementary strand after viral infection.<sup>2,23</sup> This step may not require cell division, since we



**Fig 5.** Integration of wild-type AAV into hematopoietic progenitors. DNA from 10 individual colonies was analyzed with primers specific for  $\beta$ -actin (control, below), and primers specific for the coding sequences of the AAV capsid protein. Eight colonies gave a positive signal, and two (5 and 9) were negative.

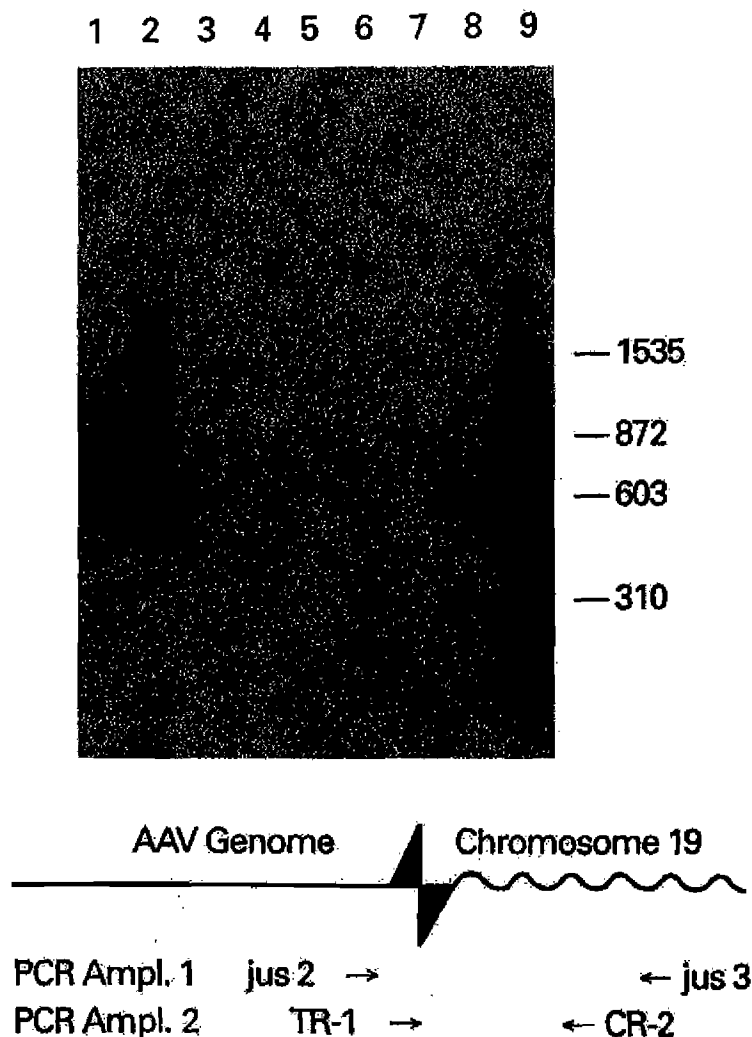


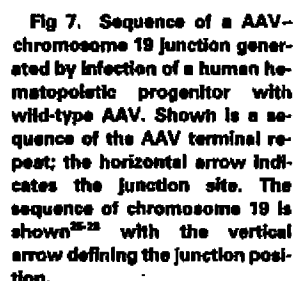
Fig. 6. Integration site analysis of the wild-type AAV genome. DNA from pools of colonies or from single colonies was amplified using primer sets that span the potential junction between the AAV genome and the chromosome 19. Lanes 1, 2, 5, and 6 show DNA from pools of 40 to 80 individual colonies. Lanes 3 and 4 show DNA from single colonies. Lane 7 shows nonradioactive gel markers that gave the calibration lengths listed on the right. Lanes 8 and 9 show DNA from cell lines latently infected with the wild-type AAV genome. The multiple bands in lane 8 are thought to arise because the intact inverted terminal repeat present in this cell line causes formation of several discrete PCR products. Amplification was performed with nested primers that span the putative integration junction as shown in the diagram.

have recently observed transduction of  $\beta$ -gal in vivo in rat brain cells using AAV vectors (X. Xiao and R.J. Samulski, unpublished observation). Subsequent integration and, therefore, persistence of the AAV genome may not occur in all infected cells. Indeed, we found that most Detroit 6 cells expressed a reporter gene shortly after infection, but only a minority appeared to exhibit continued expression of the rAAV genome.

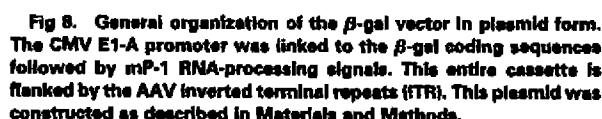
Very sensitive PCR assays can detect low levels of AAV DNA unassociated with cells in methylcellulose culture medium (unpublished observations), presumably because the virus particles to which the progenitors were exposed are stable during 10 to 14 days of incubation. These technical concerns distinguish analysis of rAAV from retroviral vectors where detection of proviral DNA can be equated with genome integration.<sup>2</sup> We scored colonies as positive only if the signal intensity was comparable to a cellular gene control (Fig 2). rAAV has recently been reported to infect murine clonogenic hematopoietic progenitors.<sup>41</sup> Neo<sup>R</sup> colonies were observed and viral DNA was demonstrated in a proportion of these colonies by PCR analysis. Although these data are

interesting and potentially important, there are a number of issues of concern. First, the ability of cells to grow for several days in G418 does not establish integration of the neo<sup>R</sup> gene. Second, control amplification of a cellular gene was not performed, so that there was no calibration of the rAAV PCR-derived signal to verify that the genome was present in single-copy equivalence. In our experiments, we could not verify expression from the transduced rAAV genome using a reverse transcription-PCR (RT-PCR) because the spliced RNA species predicted by the small intron in the proviral genome (Fig 8) could not be detected (data not shown). In recent experiments, we have used a rAAV vector containing a mutationally marked human  $\gamma$ -globin gene, linked to elements from the  $\beta$ -globin gene cluster locus control region, to transduce human erythroid progenitors. Spliced globin mRNA derived from the rAAV-transduced gene was present at high levels in the majority of colonies (J. Miller and A. Nienhuis, unpublished observations), confirming the ability of rAAV to transduce human hematopoietic progenitors with high frequency.





Despite the remaining technical problems, rAAV appears



...CAGCAGGAGGCAAAAGCGCGGGCGCTCGGGCGAGCTTTGGTATCAGCGCGCTGCAACCAAGTCAAGCGCGCGCGCGCG...

to be a highly promising vector system. Previously, we had established that integration of one to two unrearranged genomes occurs in the majority of latently infected cells.<sup>31,32</sup> AAV's nonpathogenicity and potential for preferred chromosomal integration sites makes it a highly attractive vector system for gene therapy applications. Our demonstration that hematopoietic progenitors are infected with high efficiency supports future efforts to develop this vector system.

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